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Inhibitory Regulation of Adenylyl Cyclases. Evidence Inconsistent with $\beta\gamma$-Complexes of G_i Proteins Mediating Hormonal Effects by Interfering with Activation of G_s

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The possible effect of cholera toxin (CTX) on hormonal inhibition of adenylyl cyclase in somatostatin (SST)-sensitive GH_3 cells was quantitatively evaluated. The toxin treatment employed led to an essentially complete ADP ribosylation of all α_s subunits of the stimulatory regulatory component (G_s) of the system and to *ca.* 5- to 7-fold increases in the activity measured, yet it failed to affect the inhibitory action of SST regardless of whether analyzed in terms of degree of inhibition (*ca.* 60%) that is attainable or in terms of the apparent K_{act} with which the inhibitory hormone elicits its action. In absolute terms the activity inhibited after CTX was *ca.* 6 times larger than that inhibited under control conditions, indicating that SST is equally effective in regulating control and CTX-stimulated adenylyl cyclase system and that interpretations are independent of possible intramembranous compartmentalizations of adenylyl cyclase and its various regulatory components. Since CTX-mediated ADP ribosylation of the α-subunits of G_s has been demonstrated to result in an at least 10-fold decrease in the potency (*i.e.* EC_{50}) with which the $\beta\gamma$-complexes of G proteins act to stabilize preactivated purified α-subunits of G_s and in an approximately 300-fold decrease in the potency with which exogenously added $\beta\gamma$-complexes act to prevent activation of G_s in intact membranes, the present data indicate that $\beta\gamma$-complexes cannot be mediating the inhibitory effects of hormones by interfering with activation of the G_s of adenylyl cyclase. Although inhibition of adenylyl cyclase may be due to direct action of $\beta\gamma$ on the catalytic unit of the enzyme, it is suggested that, in spite of failures to experimentally demonstrate major inhibitory effects of activated α-subunits of G_i on the catalytic activity of reconstituted or variously modified adenylyl cyclase systems, α_i is nevertheless the most likely mediator of hormonal inhibition of adenylyl cyclase systems. Possible alternate roles for $\beta\gamma$-complexes in membranes are discussed. (Molecular Endocrinology 1: 669–676, 1987)

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INTRODUCTION

It is now clear that adenylyl cyclase systems,[1] which are under both positive (stimulatory) and negative (inhibitory) regulation by hormones, are formed of at least three types of components: C,[2] one or more receptors that either stimulate (R_s) or inhibit (R_i) the system, and two intervening nucleotide-binding coupling proteins: G_s, activated by R_s-type receptors and stimulatory to C, and G_i, affected by R_i-type receptors and inhibitory to the system (1–3). In intact membranes, G_i may exist in a 5- to 10-fold molar excess over G_s (4).

[1] The term adenylyl cyclase (ATP pyrophosphate-lyase (cyclizing): E.C. 4.6.1.1), instead of adenylate cyclase is used throughout. We do this because it describes correctly the fact that the enzyme catalyzes the cyclization of the adenyl-5′yl moiety with release of inorganic pyrophosphate and H^+. The enzyme does not catalyze the cyclization of the adenylate moiety. This would require release of a pyrophosphoryl group and of the 3′-hydroxyl group of the ribose, which does not occur (41).

[2] The abbreviations and trivial names used are: C, catalytic unit of adenylyl cyclase systems; CCh, carbamylcholine; GMP-P(NH)P, guanyl-5′yl imidodiphosphate; GTPγS, guanosine 5′(γ-thio)triphosphate; G, nucleotide regulatory components of adenylyl cyclase systems; G_s, stimulatory G; G_i, inhibitory G; R: hormone receptor; R_s, stimulatory R; R_i, inhibitory R; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; SST, somatostatin; VIP, vasoactive intestinal peptide; CTX, cholera toxin.

One of the currently most intriguing aspects of adenylyl cyclase regulation is the role of the inhibitory regulatory protein G_i and the mode of action of hormone receptors that couple to the G_i, for it is uncertain how G_i acts. This uncertainty arose as a result of: 1) the discovery of the subunit structure of both G_s and G_i, which are $\alpha\beta\gamma$-heterotrimers differing only in their α-subunits (5); 2) indications that activation of both G proteins is associated with a subunit dissociation reaction according to $\alpha\beta\gamma \xrightarrow{G,Mg} \alpha^{*G} + \beta\gamma$ (6–8); and 3) the finding that exogenous addition of $\beta\gamma$, the product of the activation reaction of G_i, is inhibitory to the activation reaction of G_s by what is thought to be a mass action effect (9). Thus, inhibitory hormones may reduce the overall adenylyl cyclase activity either by causing a local increase of $\beta\gamma$-subunits which in turn could reduce the steady state level of activated α_s, or by formation of activated α_i^* subunits which could reduce the cyclizing activity of the system via direct interaction with the catalytic unit C of the system. Indirect data exist to support both possibilities. The $\beta\gamma$-mediated mode of action of inhibitory hormones was suggested by Katada *et al.* (10) who found that addition of the $\beta\gamma$-subunit complex to platelet membranes results in reduction of cyclizing activity, while addition of the resolved α_i-subunit resulted in stimulation of cyclizing activity. Also, Cerione *et al.* (11) were able to show reduction of cyclizing activity in phospholipid vesicles only when C was reconstituted with G_s, but not when it was alone. It was proposed that hormonal inhibition of adenylyl cyclase is due to to a mass action effect of $\beta\gamma$-subunits originating from G_i and acting to inhibit activation of G_s (10). On the other hand, an α_i-mediated mode of action of G_i (12–15), as well as of R_i-type receptors (16), was suggested by experiments with *cyc*$^-$ S49 lymphoma cell membranes [deficient in α_s (17)], in which both nucleotide as well as nucleotide-dependent hormone-stimulated inhibition of activity was observed, and in which the percent inhibition of activity attainable by activation of the G_i pathway remained unaltered by exogenous addition of excess preactivated G_s (15).

Both G proteins are ADP-ribosylated at their α-subunits by bacterial toxins. The toxin affecting each G and the functional consequences differ, however. G_i is affected by pertussis toxin and this leads to a loss of both its capacity to interact with R_i-type receptors (18, 19) and its susceptibility to become activated by GTP, while retaining its response to become activated by nonhydrolyzable GTP analogs such as GMP-P(NH)P (12). G_s, on the other hand, is affected only by CTX, the ADP-ribosylated G_s being activated by GTP almost as effectively as by nonhydrolyzable analogs such as GTPγS or GMP-P(NH)P (20, 21).

Using $\beta\gamma$-induced stabilization of preactivated G_s or $\beta\gamma$-mediated inhibition of G_s activation by GTP plus forskolin as bioassays, Kahn and Gilman (22) showed that CTX reduces the potency of $\beta\gamma$ by 10- or 300-fold, respectively. This indicated that ADP ribosylation of α_s not only increases the effectiveness of GTP to activate G_s, but also decreases the affinity of the α_s-subunit for $\beta\gamma$. It was concluded that ADP-ribosylation facilitates, by this mechanism, the subunit dissociation reaction (22).

In the present study we have made use of this interesting observation to probe whether hormonal inhibition of adenylyl cyclase can indeed be accounted for by deactivation of G_s via $\beta\gamma$ from G_i in a complete $G_s/G_i/C$ system, by testing for an effect of CTX on an G_i-dependent inhibition of adenylyl cyclase. Thus, if we accept that hormone-activated G_i acts primarily by deactivating G_s via $\beta\gamma$, we reasoned that the CTX-mediated reduction in the effectiveness of $\beta\gamma$ to inhibit G_s-dependent adenylyl cyclase activity should decrease the actions of hormones acting through G_i or, at least, cause their dose-response curves to be shifted toward higher concentrations in a manner proportional to the CTX-mediated decrease in affinity of $\beta\gamma$ for α_s. We found that, although absolute activities are markedly enhanced in CTX-treated membranes as compared to control membranes, the effectiveness and dose-response relationships with which inhibitory hormones act are unaffected.[3] Implications of this finding are discussed.

RESULTS

Completeness of Action of CTX

Autoradiograms of homogenate proteins analyzed by SDS-PAGE after [^{32}P]ADP-ribosylation with CTX (Fig. 1), indicated that GH_4C_1 cells contain two substrates for the toxin: one of apparent M_r = 42,000 and the other of apparent M_r = 52,000, corresponding to the type 1 and type 2 α_s-subunits of G_s proteins, which are both thought to mediate stimulatory effects of R_s-type receptors (25). Such autoradiograms showed further than an overnight treatment of cell cultures with 1.6 μg/ml CTX results in an essentially complete disappearance of sites susceptible to subsequent derivatization with [^{32}P]ADP-ribose by 100 μg/ml CTX. This result demonstrated that the conditions chosen for treatment of intact cells with CTX showed in essentially complete modification of all G_s molecules and that the properties of the adenylyl cyclase system of such treated cells should therefore be those of the fully intoxicated systems as opposed to representing those of mixture of unaffected and affected systems, in which the former could constitute a significant proportion of the total.

Effect of CTX on Adenylyl Cyclase Activities

The conditions of assay used and the concentration range of homogenate protein added to measure adenylyl cyclase activities were such that accumulation of cAMP was both linear with time for periods up to 20–

[3] Preliminary experiments carried out with CTX-treated human platelet membranes, showing the same results as presented here with membranes from GH_4C_1 cells, were presented (23, 24).

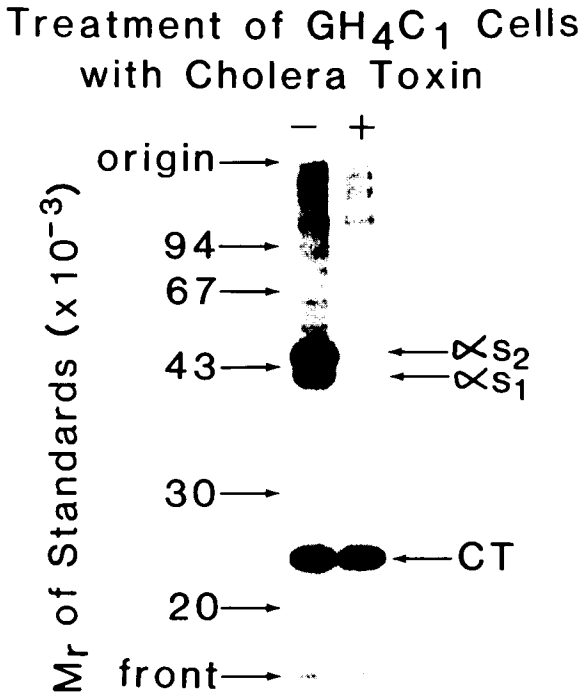

Fig. 1. ADP Ribosylation of α-Subunits of G_s by CTX in Intact GH_4C_1 Cells

Cells were treated or not with 4 μg/ml CTX for 15 h, harvested, homogenized, and the resulting homogenates were subjected to a secondary test ADP-ribosylation by 100 μg/ml CTX in the presence of [^{32}P]NAD$^+$. The resulting mixtures were then analyzed for incorporation of [^{32}P]ADP-ribose into unaffected α_s-subunits by SDS-PAGE followed by autoradiography. A photograph of the autoradiogram is shown, as are apparent molecular weights ($\times 10^{-3}$) of standards. α_{s1} and α_{s2}, α-subunits of G_s; CT, self-ADP-ribosylated A subunit of CTX.

30 min[4], and proportional to the amounts of homogenate protein added (not shown). Figure 2 illustrates the type of activities obtained when homogenates from control and CTX-treated cells were assayed without or with guanine nucleotide [GTP or GMP-P(NH)P], NaF, or forskolin (plus ethanol) addition. In this and eight similar experiments, addition of 20 μM GTP to homogenates from control cells caused a minor (*ca.* 20–30%) increase in activity over that measured without its addition and 10 μM GMP-P(NH)P caused an approximately 2-fold stimulation. NaF was a more effective stimulator than GMP-P(NH)P, increasing activity by 5- to 6-fold, but was still much less effective than forskolin. This diterpene, assayed in the presence of 20 μM GTP, stimulated the activity 20- to 25-fold to absolute values of 400–500 pmol cAMP formed/min·mg protein (n = 9). As expected, treatment of cells with CTX resulted in a marked enhancement of activity by endogenously present GTP to values that ranged from 200–230 pmol cAMP formed/min·mg protein. Exogenous addition of 20 μM GTP to homogenates of toxin-treated cells consistently resulted in a slight (10–15%) inhibition of activity and addition of 10 μM GMP-P(NH)P or 10 mM NaF led to 60–70% or 40–50% inhibition of activity, respectively, reflecting the activation of the G_i pathway by these ligands (14, 26). Forskolin-stimulated adenylyl cyclase activity was slightly decreased by CTX treatment: absolute activities in nine such experiments ranged from 350–380 pmol cAMP formed/min·mg.

[4] Except for progress curves with GMP-P(NH)P, which displayed characteristic lag phases (43, 44).

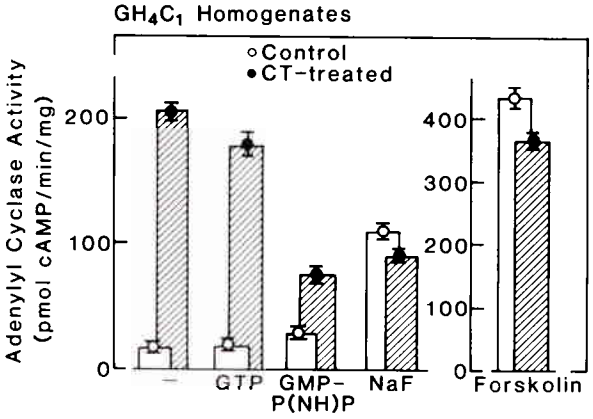

Fig. 2. Effect of Addition of GTP, GMP-P(NH)P, NaF, and Forskolin on Adenylyl Cyclase Activity in Homogenates of Control and CTX-Treated GH_4C_1 Cells

Results are the means ± SD of triplicate determinations. □, Control cells; ▨, toxin-treated cells.

Under the assay conditions chosen (~1 mM free Mg^{2+}), the effects of stimulatory (VIP) and inhibitory (SST and CCh) hormones were easily observed in homogenates of control cells (Figs. 3 and 4), the inhibitory effect being equally visible in the absence and the presence of stimulatory regulation by VIP. Treatment of cells with CTX interfered little with the degree to which inhibitory hormones act, the percentage of the inhibition they mediate being approximately the same in homogenates of control and toxin treated cells. However, the stimulatory action of VIP was essentially abolished by CTX, this being clearly due to the facilitation of the activation of the system by GTP caused by the toxin, leaving little room for further hormonal stimulation of GTP action. This is illustrated further in Fig. 3, in which the effect of VIP on adenylyl cyclase in homogenates of control and toxin-treated cells was studied as a function of its concentration. The apparent K_{act} for VIP stimulation of activity ranged from 2–4.5 nM (n = 3).

Effect of CTX on Concentration-Effect Curves of Inhibitory Hormones

The results described above indicated clearly that toxin treatment 1) facilitates activation of G_s, and 2) does not interfere with inhibitory regulation by hormones, when analyzed in terms of percentage of control. Figure 4 illustrates the concentration-effect curves for SST assayed in the absence and presence of a saturating concentration of VIP in homogenates of control and toxin-treated cells. IC_{50} values for SST ranged in various experiments from 6–11 nM in the absence of VIP and

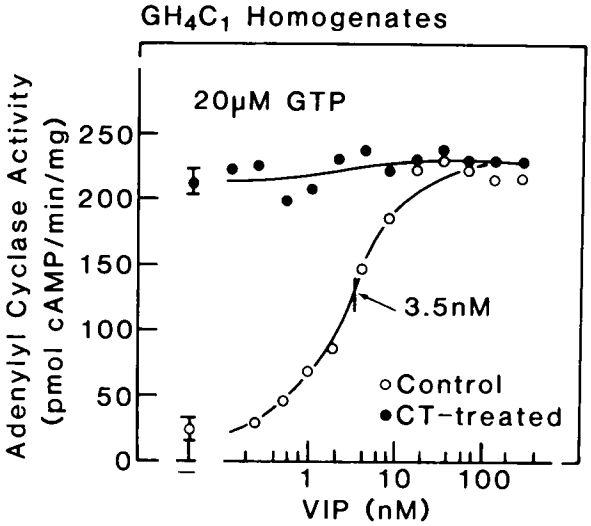

Fig. 3. Concentration-Effect Curves for the Stimulatory Action of VIP on Adenylyl Cyclase Activity in Homogenates of Control and CTX-Treated Cells

Values at zero concentrations of VIP are means of triplicate ± SD; the remainder of the points are from single incubations. ○, Control cells; ●, toxin-treated cells. Twenty micromolar GTP was present throughout.

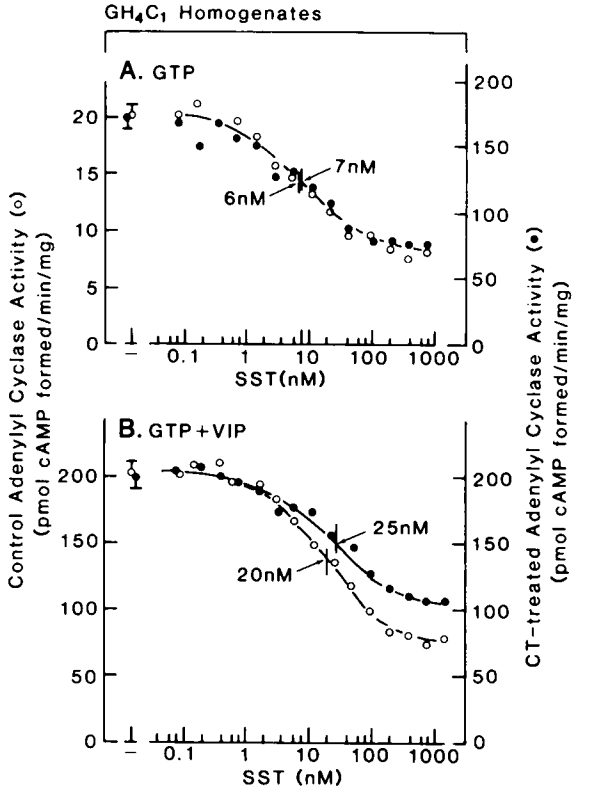

Fig. 4. Concentration Effect Curves for the Inhibitory Action of SST on GTP-Stimulated and VIP plus GTP-Stimulated Adenylyl Cyclase Activities in Homogenates of Control and Cholera Toxin-Treated Cells

Values at zero concentrations of SST are means of triplicate ± SD; the remainder of the points are from single incubations. ○, Control cells; ●, toxin-treated cells. Twenty micromolar GTP was present throughout.

17–25 nM in the presence of VIP, and indicated that a somewhat higher concentration (1.7- to 3-fold) of SST is needed to obtain a half-maximal effect in the presence of the G_s-stimulating hormone than in its absence. However, and as illustrated in Fig. 4 for SST and Fig. 5 for CCh, on comparing the IC_{50} values for SST or CCh obtained in any given experiment with homogenates from control cells to those from CTX-treated cells, no effect of CTX on the IC_{50} values was detected.

DISCUSSION

Inhibition of the G_s defective *cyc*⁻ S49 cell membrane adenylyl cyclase by guanine nucleotides was first reported in 1982 (13). The inhibition was subsequently shown to be G_i-mediated (12) and under hormonal control (16). Normally, data such as these would be interpreted as indicating that hormonal inhibition of adenylyl cyclase activity is a process that can proceed independently of presence of a functional G_s, and that even when a functional G_s is present, the basic mechanism by which hormonal inhibition comes about should still be a reflection of the mechanism that operates in its absence. However, direct experiments testing for an inhibitory effect of the nucleotide-binding subunit of G_i, the protein responsible for mediating hormonal inhibition of the system, have either failed or been rather disappointing in their magnitude (9–11). Instead, experiments with pertussis toxin-treated membranes, comparing effects of the nucleotide binding component of G_i to those of the $\beta\gamma$-complex of the molecule, showed marked effects of the latter and only minor or no effects of the former (9, 10). On the basis of such experiments, it was proposed (9, 10, 27), that "inhibitory receptors act by effecting dissociation of subunits, with inhibition of activity being mediated by the $\beta[\gamma]$complex, and that the stimulatory and inhibitory pathways that control

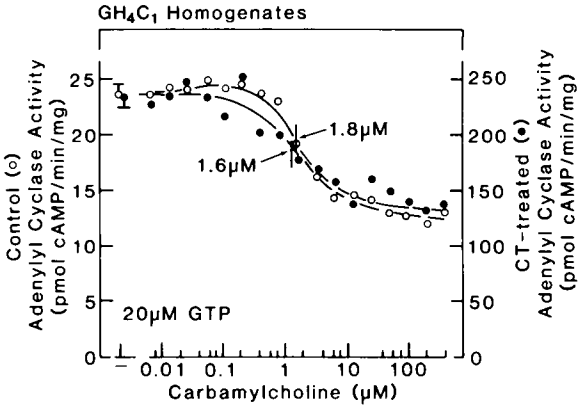

Fig. 5. Concentration Effect Curves for the Inhibitory Action of the Acetylcholine Receptor Ligand CCh on GTP-Stimulated Adenylyl Cyclase Activities in Homogenates of Control and CTX-Treated Cells

Values at zero concentrations of CCh are means of triplicate ± SD; the remainder of the points are from single incubations. ○, Control cells; ●, toxin-treated cells. Twenty micromolar GTP was present throughout.

adenylyl cyclase activity interact *primarily* through their common $\beta[\gamma]$complexes at the level of the guanine nucleotide regulatory components" (*cf.* Ref. 27). Failure to obtain inhibitory regulation of the catalyst C by G_i on insertion into reconstituted vesicles (11), would seem to support this proposal and, implicitly, questions further what the meaning of results obtained with *cyc*$^-$ membranes might be. Clearly, the role or roles of $\beta\gamma$-complexes in membranes remained enigmatic. Specifically, it was not known whether they are indeed intrinsically inhibitory to the expression of G_s activity in a membrane environment, and if so, whether they qualify as the primary mediators of hormonal inhibition.

Since most of the data mentioned above had been obtained either with *cyc*$^-$ membranes, or with membranes subjected to pretreatments with pertussis toxin to block effects of hormones on endogenous G_i (9, 10), we now sought to gain new information using a system that would involve neither the *cyc*$^-$ membrane nor the use of pertussis toxin. The experiments presented here, therefore, used GH_4C_1 cells, a non-*cyc*$^-$ membrane system, in which dual regulation of adenylyl cyclase is very prominent under the assay conditions used, and this system was probed with CTX, leaving the operation of G_i unaltered. We tested whether conditions known to alter sensitivity of G_s to $\beta\gamma$-complexes would result in a concomitant alteration in the sensitivity of the dually regulated adenylyl cyclase system to inhibitory hormones, as predicted if the primary mode of action of inhibitory hormones were to decrease the steady state level of active G_s by local increases in $\beta\gamma$-complexes.

It is clear from the data presented, however, that a treatment that both facilitates G_s activation by GTP (20, 22) and decreases the inhibition of this activation by the $\beta\gamma$ complex of the signal transducing proteins (22), affects neither the potency nor, to any significant extent, the efficacy with which receptor ligands act to stimulate G_i and cause inhibition of adenylyl cyclase activity.

The question may be asked as to whether the inhibitory regulation observed after toxin treatment indeed reflected inhibition of ADP-ribosylated G_s/C complexes and not regulation of traces of unaffected systems. We do not think this to be so, for three reasons. First, our probing for presence of unaffected G_s in homogenates of toxin-treated cells as seen by [^{32}P]ADP-ribosylation failed to reveal even traces of such unaffected G_s (Fig. 1). Second, our probing for presence of functionally active unaltered G_s as seen by existence of VIP-mediated activation of G_s by GTP beyond that caused by GTP alone also failed to reveal any significant amount of unaffected G_s (Fig. 3). Third, the percent inhibition of activity (60–70%) obtained with either SST or CCh in homogenates of control and CTX-treated cells was unaltered when assays were performed in the absence of a stimulatory hormone (Figs. 4A and 5). Since the absolute activities under these assay conditions differed by a factor of 5- to 10-fold between membranes from control and toxin cells, such that the activity inhibited by hormone after toxin treatment was 6 to 7 times the activity obtainable without either SST or CCh in the untreated system, there cannot be any doubt that inhibition of activity in toxin treated membranes is indeed inhibition of ADP-ribosyl-G_s/C complexes as opposed to inhibition of any remainder of unmodified G_s/C complexes.

On the basis of the above, we conclude that hormonal inhibition of adenylyl cyclase cannot be the primary result of a local increase of $\beta\gamma$-complexes acting by mass action to interfere with normal regulation of G_s. By inference, and in agreement with data obtained with the *cyc*$^-$ system, G_i-mediated inhibition of adenylyl cyclase must actively involve the interaction of the adenylyl cyclase proper with the activated G_i. Although we feel that the subunit mediating the inhibitory action is α_i (28), the experiments do not rule out the possibility that $\beta\gamma$-dimers may be the inhibitors as suggested by Katada *et al.* (29).

We reported recently that optimal stimulation of an R_s/G_s/C system inserted into reconstituted phospholipid vesicles by hormones, *i.e.* attainment of ratios of stimulated activity relative to that in the absence of agonist in the order of 5 to 7, requires not only occupancy of the R_s-type receptor by its specific agonist but also the previous attenuation of G_s/C activity by $\beta\gamma$-complexes, added as such or in the form of a combination of a G_i and an R_i-type receptor (30). These studies indicated not only that $\beta\gamma$-complexes are indeed inhibitory to G_s in the membrane environment, in agreement with data obtained with pertussis toxin-treated membranes (9), but also that hormonal stimulation of activity involves, in fact, stimulation of G_s plus release of G_s from inhibition by excess ambient $\beta\gamma$-complexes (30). Thus, it was found that only minor stimulations of R_s/G_s/C systems are obtained (31–33) in the absence of $\beta\gamma$-complex addition, other than that unavoidably added as a contaminant of the rather high levels of G_s used in such assays (4, 32), or of activated G_i, which is presumably cycling through activation-deactivation and dissociation-reassociation cycles and therefore generating $\beta\gamma$-complexes *in situ* under the influence of GTP (34, 35). It can be concluded, therefore, that very likely a "normal" adenylyl cyclase system assayed under basal conditions of GTP and Mg ion, is under constant tonic inhibition of relatively saturating concentrations of $\beta\gamma$-complexes. This provides an independent, albeit circumstantial argument against the role of $\beta\gamma$-complexes as active mediators of inhibitory effects of hormones.

It may be of interest to remark that the inhibitory regulation of adenylyl cyclase by NaF and nonhydrolyzable guanine nucleotides, such as GMP-P(NH)P, seen very notably in the *cyc*$^-$ system, is also operative in "complete" adenylyl cyclase systems, such as that of the GH_4C_1 cell employed in the studies presented here. This became especially evident after treating the cells with CTX, which resulted in essentially full activation of the G_s by the cellular GTP present in the homogenates assayed for activity and led to the "uncovering" of the marked inhibitory effect of NaF and GMP-P(NH)P. Partial stimulations obtained with NaF and GMP-P(NH)P in control cell homogenates are therefore the result of their dual effects to activate stimulatory G_s and inhibitory G_i at the same time.

MATERIALS AND METHODS

Cells

GH_4C_1, a clonal cell line derived from a rat PRL-secreting pituitary tumor (36), was kindly given to us by Dr. Armen Tashjian, Jr. (Laboratory of Toxicology, Harvard School of Public Health, Boston, MA) and was grown in monolayer culture to close to confluency in Ham's F10 medium supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Routinely, cells were split to a ratio of 1–5, grown under 20 ml medium in Falcon T75 plastic culture bottles at 37 C in an atmosphere of 95% oxygen 5% CO_2 for 4 days, treated or not for an additional night (*ca.* 16 h) with 1.6 μg/ml CTX and harvested by strong shaking into the growth medium. The cell suspensions were centrifuged at 1000 to 1200 × *g* at 4 C for 15 min, washed once with a balanced salt solution (0.01% glucose, 5 μM $CaCl_2$, 98 μM $MgCl_2$, 0.54 mM KCl, 126 mM NaCl, 14.5 mM Tris-HCl, pH 7.6) and resuspended in 2.0 ml cold homogenization buffer [27% (wt/wt) sucrose, 1 mM EDTA, and 20 mM Na-HEPES, pH 7.8]

Homogenization of Cells

Cells obtained in this manner (*ca.* 9–10 × 10^6 per flask) were homogenized at 0–4 C by nitrogen cavitation using a 75-ml Parr bomb: the cells were equilibrated for 15 min at 750 psi N_2 and followed by slow release into a 13 × 100-mm glass test tube. The homogenates obtained in this manner (0.7–0.9 mg protein/ml) were kept on ice and assayed for adenylyl cyclase activities within 30 min.

Adenylyl Cyclase Assays

Unless indicated otherwise, incubations were at 32 C for 10 min in a final volume of 50 μl containing 0.1 mM [α^{32}P]ATP (*ca.* 10^7 cpm/assay), 2.0 mM $MgCl_2$, 1.0 EDTA, 1.0 mM [^{3}H]cAMP (*ca.* 15,000 cpm/assay), 0.1% BSA, a nucleoside triphosphate regenerating system composed of 20 mM creatine phosphate, 26 U/ml creatine kinase, 25 U/ml myokinase, 25 mM Tris-HCl, pH 7.6, 10 μl homogenate and when present: 20 μM GTP, 10 μM GMP-P(NH)P, 100 μM forskolin plus 0.5% ethanol, 10 mM NaF, 1 μM VIP and the indicated concentrations of SST or CCh. The reactions were stopped and the [^{32}P]cAMP formed was assayed by a modification (37) of the method of Salomon *et al.* (38).

Activation of CTX for ADP-Ribosylation

CTX was activated for 30 min at 32 C at a concentration of 3.0 mg protein/ml in a final volume of 40 μl containing 50 mM dithiothreitol (DTT) and 0.1% SDS. This mixture was then diluted 5-fold with medium containing BSA and Tris-HCl, pH 7.6, to give a final concentration of 0.05% and 10 mM, respectively.

ADP-Ribosylation of N_s in Homogenates

Ten microliters of appropriately diluted homogenates to give a concentration of 0.5 mg protein/ml were incubated in a final volume of 60 μl with 30 μl 2.0 mM GTP, 2.0 mM ATP, 20 mM $MgCl_2$, 600 mM K_2HPO_4/KH_2PO_4, pH 8.0, 2 mM EDTA, 20 mM thymidine, and 45 mM Tris-HCl, pH 7.6, 10 μl [^{32}P]NAD^+ (10^7 cpm; SA, 100–300 Ci/mmol) and 10 μl 0.05% BSA, 10 mM Tris-HCl, pH 7.6, 10 mM DTT, and 0.02% SDS, containing or not 600 μg CTX/ml. After incubating for 40 min at 32 C, the reactions were stopped by addition of 1.0 ml ice-cold 20% trichloroacetic acid. The samples were then mixed, allowed to stand on ice for 10 min, and centrifuged in a table top centrifuge at 2000 rpm (*ca.* 1000 × *g*) for 30 min in the cold. The supernatants were carefully aspirated and discarded. The remaining traces of trichloroacetic acid were removed by overlaying the pellets with 1.5 ml ethyl ether followed by a another centrifugation at 2000 rpm in the cold and removal of the ether by aspiration. The tubes were then placed in a 32–37 C water bath to evaporate the remaining ether. The samples were analyzed for incorporation of [^{32}P]ADP-ribose into α_s-subunits of GH_4C_1 cells by resuspending in 20 μl Laemmli's sample buffer (39) at room temperature followed by SDS-PAGE (39) and autoradiography as described (5).

Materials

[α-^{32}P]ATP, synthesized according to Walseth and Johnson (40), and [^{32}P]NAD^+, synthesized according to Cassel and Pfeuffer (41) with modifications,[5] were supplied by the Molecular Endocrinology Core Laboratory of the Baylor College of Medicine Diabetes and Endocrinology Research Center. CTX was purchased from List Biologicals (Campbell, CA). Ham's F10 medium (with L-glutamine and prepared as recommended by the manufacturer), fetal calf serum, and a mixture of penicillin and streptomycin were purchased from GIBCO (Grand Island, NY). VIP, SST, and CCh were from Sigma Chemical Co. (St. Louis, MO). All other reagents were of the highest commercially available purity and were used without further treatment. As a result, and because adenylyl cyclase assays were carried out with total homogenates, these assays probably contained up to 1 μM GTP (21, 42).

Reproducibility of Results and General Design of Experiments

All experiments referred to in this paper were carried out least twice and most were done three times. In all instances in which the effects of CTX were explored, homogenates of control and CTX-treated cells were analyzed in parallel and on the same day for both adenylyl cyclase activities and susceptibility of α_s-subunits of G_s to be [^{32}P]ADP-ribosylated by 100 μg/ml CTX. This documented the degree of effectiveness of each of the toxin treatments.

Acknowledgments

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